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(54) Title: STRAIN OF BACTERIA OF THE SPECIES *LACTOBACILLUS PARACASEI* SUBSP. *PARACASEI*, COMPOSITION THEREOF FOR USE IN FOOD AND PRODUCT CONTAINING SAID STRAIN

## (57) Abstract

Strain of *Lactobacillus* useful as probiotics in food and naturopathic medicines and which is resistant *in vitro* against hydrochloric acid and gastric juices and tolerates bile salts without deconjugating them whereas strong assimilation is occurring and which has good survival at the passage through the stomach and the gastrointestinal tract and which strain is growing optimally at about 37 °C, which strain is *Lactobacillus paracasei* subsp. *paracasei*, which is a Gram-positive, homofermentative, rod-shaped bacterium capable of producing L-lactic acid and containing three plasmids having a size of 2.2, 4.36 and 9.1 Kb, respectively. The invention also relates to a composition containing the strain and a product consisting of or containing a concentrate of the strain.

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STRAIN OF BACTERIA OF THE SPECIES *LACTOBACILLUS PARACASEI* SUBSP. *PARACASEI*, COMPOSITION THEREOF FOR USE IN FOOD AND PRODUCT CONTAINING SAID STRAIN

The present invention relates to a strain of *Lactobacillus paracasei* subsp. *paracasei*, a composition thereof for use in food as well as a product containing said strain.

## Definition and Characterisation of the Strain

The novel strain (which in the following for simplicity will be designated LMG P-17806) is a variant of the species *Lactobacillus paracasei* subsp. *paracasei*. It has the characteristics of the species with a GC-content of 44 %. LMG P-17806 has been isolated from samples from the gastrointestinal micro-flora of humans. LMG-P-17806 is a Gram-positive, homofermentative rod-shaped bacteria. It produces L-lactic acid (laevorotatory stereoisomer of lactic acid) and grows optimally at 37°C. The strain is characterised by being tolerant in-vitro against hydrochloric acid and gastric juice by tolerating bile salts without deconjugating them and by having a great ability of assimilating cholesterol. The strain is also characterised by containing three plasmids having a size of 1.1, 4.36 and 3.1 Kb respectively. Other characteristics are that the strain is fermenting ribose, adonitol, galactose, glucose, fructose, mannose, sorbose, mannitol, sorbitol, N-acetyl-glucosamine, esculin, cellobiose, maltose, lactose, sucrose, trehalose, inulin, melezitose, D-turanose and D-tagatose. On the other hand it does not ferment glycerol, erythritol, D- and L-arabinose, D- and L-xylose,  $\beta$ -methyl-D-xyloside, rhamnose, dulcitol, inositol,  $\alpha$ -methyl-D-mannoside,  $\alpha$ -methyl-D-glucoside, amygdalin, arbutin, salicin, melibiose, raffinose, starch, glycogen, xylitol, gentiobiose, D-fucose, D-lyxose, L- and D-fucose, D- and L-arabinose and D- and L-Ketogluconate.

The strain has been characterised by SDS gel electrophoresis, in which it has been compared to six other strains of *Lactobacillus paracasei* subsp. *paracasei*, nine are accompanying figure. In this comparison it has been shown to differ from all.

other described strains and at the same time as it when being compared to other *Lactobacilli* appears to belong to the designated species. It has also been characterised with regard to ribosomal RNA in a so called Riboprinter®. With this method the strain has been shown to possess 76% similarity with the type strain for *Lactobacillus paracasei* subsp. *paracasei* and 72% similarity to the type strain of *Lactobacillus casei* analysed at the same occasion.

The strain has been deposited at Belgian Coordinated Collections of Microorganisms - BCCM, LMG collection, and there been given the accession No. LMG P-17606.

#### **The Advantages of the Strain**

LMG P-17606 has, when compared to known strains of *Lactobacillus*, crucial advantages in the use as probiotics in food and naturopathic medicines by a unique combination of good properties:

- the strain has good resistance against gastric juice and bile salts, but unlike many other strains it does not deconjugate the bile salts;
- it has a great ability to assimilate cholesterol;
- the strain is well managing the passage through the stomach;
- the strain has an influence on the conditions in the model of large intestine by increasing the production of L-lactic acid therein;
- the strain is not more pro-inflammatory than common yoghurt bacteria;
- the strain prevents intestinal cells from being invaded by pathogenic microorganisms, such as *Salmonella typhimurium*;
- the strain has an antagonistic action against the gastric ulcer bacterium *Helicobacter pylori*;

- the strain forms bacteriocins which are active against clostridia;
- the strain survives well in milk as well as in frozen and dried form;
- the strain, unlike most other lactobacilli has a favourable influence on the taste of fermented milk products (does not give any tang).

The present strain of *Lactobacillus paracasei* subsp. *paracasei* can be used as an additive to food or as naturopathic medicine, so called "Medical Food", or as an additive to naturopathic medicine.

Such medicines can be used for children with the purpose of alleviating atopic problems; for elderly persons in order to correct altered microflora caused by normal alterations by age or an altered secretion of hydrochloric acid; and for persons in general in order to normalise the intestine flora, whereas the content of clostridium bacteria is decreasing, lactobacilli and bifido bacteria being increased and high contents of coliformic bacteria being decreased.

By means of these properties the strain LMG P-17806 differs from previously known strains, which will be shown in the examples below.

#### **Preparation of the Strain**

The strain is prepared in the usual way for lactobacilli. A substrate suited for lactobacilli is used. This substrate should for instance contain at least one of the carbohydrates which the strain can ferment according to what is stated above, in combination with proteins, vitamins, minerals and other nutrients which normally are required by lactobacilli. Examples of suitable commercial substrates are yeast extract-glucose broth, MRS (ie Man-Poggosa-Sharp broth), Rogosa, milk added with a minor amount of a yeast extract, etc. The strain is cultivated microaerophilically or in the complete absence of

oxygene, suitably at a temperature between +15°C and +40°C. If the substrate is grafted with 0.1 to 1% of graft a culture-time of between 10 and 40 hours is suitable. The strain can, if desired, be concentrated by centrifugation or filtration, whereafter the concentrate is washed in order to remove the culture medium. The concentrate can then be frozen or lyophilized in the common way. In this way preparations of between 100 millions and 100,000 millions of living bacteria LMG P-17806 per g can be prepared. A preparation can then be used as such or re-used as an additive to food, for instance to milk or another product which gives LMG P-17806 the possibility to survive and, if desired, to grow.

## INVESTIGATIONS

### A. Investigation Concerning the Passage of the Strain Through the Gastrointestinal Tract

LMG P-17806 was cultivated in the way described above and added together with yoghurt culture to milk. A fermented product was produced by incubating the milk for five hours at +42°C. A palatable product was obtained which contained fully 100 millions living LMG P-17806 per gram of product. Healthy persons were given 3 x 200 g product daily for one week. The total intake of LMG P-17806 was between 40 billions and 200 billions.

Faeces samples were examined before the intake, after one week of consumption and one week after the intake had ceased. As is evident from Tables 1 and 2 below, a strong increase in the number of lactobacilli in the test subjects was obtained. Two isolates per test subject were classified as to species on each occasion, i.e. 20 isolates in total. 18 of the isolates for the consumption time appeared to consist of LMG P-17806 according to fenotypical classification. This bacteria strain was not discovered in the samples before or after the intake of LMG P-17806.

In average the contents of the faeces samples during the supply were very high and varied only moderately from 60 millions to

321 millions per gram, i.e. with a factor of 6. Noteworthy was, that the contents were largely the same independant of what contents were measured before the start of the experiment. After the supply had ceased the contents reverted to what seems to be natural for the test subject in question.

In Tables I and I below the content of lactobacilli in faeces was determined, in millions per gram, by plating and using the substrate Rogosa.

Table 2

| Test subject | Before the experiment | During the experiment | After the experiment |
|--------------|-----------------------|-----------------------|----------------------|
| 1            | 0.05                  | 120                   | 0.07                 |
| 2            | 0.15                  | 320                   | 0.12                 |
| 3            | 0.06                  | 97                    | 0.14                 |
| 4            | 0.009                 | 53                    | 0.02                 |
| 5            | <0.001                | 1272                  | 0.002                |

Table 2

5 test subjects with high contents of lactobacilli

| Test subject | Before the experiment | During the experiment | After the experiment |
|--------------|-----------------------|-----------------------|----------------------|
| 5            | 1.2                   | 2.9                   | 1.3                  |
| 4            | 0.7                   | 0.3                   | 0.7                  |
| 3            | 2.7                   | 1.3                   | 0.19                 |
| 2            | 4.3                   | 7.4                   | 3.1                  |
| 1            | 1.5                   | 1.11                  | 1.4                  |

The examination shows that the strain has good survival at the passage through the gastrointestinal tract.

**B. Examination Concerning the Formation of L-lactic Acid by the Strain in a Model of Large Intestine**

The fermented product above was added to a so called SHIME-reactor which is an *in vitro* model of the intestine. Samples were taken from the part of the reactor which corresponds to the most important parts of the large intestine. Similar comparative tests were carried out with some other *Lactobacillus* strains, i.e. closely related *L. paracasei* subsp. *paracasei*. It is evident from the following Table 3 below LMG P-17806 gave a strong increase in the production of L-lactic acid, which is the very lactic acid isomer which is generated by LMG P-17806. A production of lactic acid is considered as favourable for several reasons, i.e. considering the antibacterial effect of the lactic acid as well as the fact that a lower pH is supposed to reduce the availability and formation of nitrogen compounds.

Table 3

Production of L- and D-lactic acid in a SHIME-reactor after the addition of different lactic acid bacteria  
in mg per litre reactor content

| Lactic acid<br>bacteria | Reactor 4 |    | Reactor 5 |     | Reactor 6 |    |
|-------------------------|-----------|----|-----------|-----|-----------|----|
|                         | L         | D  | L         | D   | L         | D  |
| LMG P-17806             | 300       | 80 | 500       | 100 | 280       | 90 |
| <i>L. paracasei</i>     | 40        | 80 | 200       | 70  | 130       | 40 |
| <i>L. rhamnosus</i>     | 30        | 10 | 10        | 70  | 80        | 10 |
| <i>L. plantarum</i>     | 50        | 30 | 60        | 70  | 40        | 50 |

In the table "L" refers to the laevo-rotatory isomer of lactic

acid and "D" to the dextrorotatory isomer.

Reactor 4 corresponds to the upper part of the large intestine, reactor 5 to the middle part and reactor 6 to the lower part of the large intestine. The investigation shows that the strain is forming L-lactic acid in the model of the large intestine.

#### C. Investigation of How the Strain is Protecting Intestinal Epithelium Cells from Invasion of *Salmonella Typhimurium*

Intestinal epithelium cells of the type CaCo-2 cells were  
cultivated in-vitro. These were added with a combination of  
lactobacilli and salmonella typhimurium in the ratio 1:1  
with the addition of 1 million salmonella per ml. The effect  
was studied after incubation for 120 min at 37°C. The amount  
of invading salmonella was determined by washing the plates  
with adhering CaCo-2 cells three times. The adhering cells  
were treated with the antibiotic gentamycin in a  
concentration of 100 mg/l for one hour in order to kill all  
bacteria which had not invaded cells. Then the plates were  
washed with PBS in order to remove all gentamycin and finally  
the entrapped bacteria were released by treating the CaCo-2  
cells with 0.1 % Triton-X during shaking. The number of  
salmonella was then determined by common plating methodology.  
LMG P-17806 had a pronounced effect in that it reduced a  
number of invaded cells. The closely related paracasei-variant  
506 on the other hand seemed rather to stimulate the invasion  
of salmonella bacteria. Also with regard to this property LMG  
P-17806 showed a positive effect. The results are reported in  
Table 4 below.

Table 4

## Invasion of salmonella

| Lactic acid bacteria | invading salmonella          |                           |
|----------------------|------------------------------|---------------------------|
|                      | without lactic acid bacteria | with lactic acid bacteria |
| LMG P-17806          | 7.6                          | 1.72                      |
| L. paracaseiF 506    | 2.8                          | 0                         |
| L. plantarum         | 0                            | 0                         |
| L. rhamnosus         | 0.8                          | 0.5                       |

The table shows that the strain LMG P-17806 gives a marked protection against invasion of salmonella bacteria.

5

**D. Investigation of Protection Against *Helicobacter Pylori***

In a mouse model where the mice had been infected with *Helicobacter pylori* the effect of supplying a fermented milk product with a strain of LMG P-17806 on one hand and without said strain on the other on the content of *H. pylori* measured in faeces was examined.

The mice were infected with 100 millions of the strain *H. pylori* 17374 in helical form at three occasions with an interval of one day. Then the mice were given experimental products and the content of *H. pylori* in faeces was measured by means of heparinised magnetic balls and Enzyme Immuno Assay. Three products were examined. All the fermented milk products appeared to reduce the share of *H. pylori*, but the effect was occurring considerably faster in the cases when the product contained LMG P-17806 in comparison to common yogurt and in comparison with a strain of *L. fermentum* K12, respectively.

Table 3

Content of *H. pylori* in faeces measured  
by heparinised magnetic balls and Enzyme Immuno Assay

| Product                             | before<br>intake | after<br>intake for<br>2 days | after<br>intake for<br>7 days | 7 days<br>after<br>cease of<br>intake |
|-------------------------------------|------------------|-------------------------------|-------------------------------|---------------------------------------|
| Common yoghurt                      | 1.48             | 1.86                          | 0.63                          | 0.25                                  |
| Yoghurt with<br><i>L. fermentum</i> | 1.65             | 1.62                          | 0.94                          | 1.61                                  |
| Yoghurt with<br>LMG P-17806         | 1.80             | 0.68                          | 0.61                          | 1.71                                  |

The figures in the table state the absorbency of 405 nm and  
5 are relative contents.

#### E. Examination of the Influence of the Strain LMG P-17806 on the Immunological Defence

The immunological defence system is controlled by a series of  
10 signal substances, so called cytokines. Some of these can be  
proinflammatory. The influence of LMG P-17806 on the  
production of cytokines TNF-alfa and IL-6 was compared with the  
influence of the two species contained in a yoghurt culture,  
*L. delbrueckei* subsp. *bulgaricus* and *Streptococcus*  
15 *thermophilus*. leucocytes were separated from human blood and  
added to living bacteria or bacteria killed with  
glutaraldehyde in an amount of 10 millions of leucocytes. As  
control lipopolysaccharides (LPS) from *E.-coli* were used. The  
results are reported in Table 4 below. The results show that  
20 the LMG P-17806 has the same inflammatory properties as a  
common yoghurt culture in the model used.

## TABLE 5

The influence of the strain LMG P-17806  
on the immunological defence

| Lactic acid bacteria          | TNF-alpha mg/l |        | IL-6 mg/l |        |
|-------------------------------|----------------|--------|-----------|--------|
|                               | living         | killed | living    | killed |
|                               | 12             | 11     | 0.4       | < 0.1  |
| <i>S. thermophilus</i> , B584 | 12             | 11     | 0.4       | < 0.1  |
| <i>S. thermophilus</i> , B581 | 1              | 1      | 0.5       | 0.2    |
| LMG P-17806                   | 1              | 1      | 0.7       | 0.1    |

**F. Examination of the Resistance of the Strain LMG P-17806  
Against Antibiotics**

Probiotics can be useful for use in connection with disorders in the balance of the intestine flora during medication with antibiotics. At the same time it is important that probiotics do not contribute to spreading of resistance to antibiotics, and this is especially important in the contemporary use of probiotics and antibiotics. The resistance of the strain LMG P-17806 against different antibiotics has been established for that reason. The sensitivity of the strain LMG P-17806 to different antibiotics were determined by establishing the content at which a reduction in the growth of the strain by 50 %, measured as optical density, was obtained.

It appeared that the strain LMG P-17806 was resistant against vancomycin and was not inhibited even by 256 mg/l. The strain showed some resistance against trimethoprim and cefotaxime, an optical density OD of 50% at 11 and 4 mg/l, respectively being obtained. The strain LMG P-17806 was on the other hand sensitive to chloramphenicol, erythromycin, rifampicin and tetracycline, where already levels below 1 mg/l resulted in an inhibition of the growth by 50%.

The influence by antibiotics was also examined as a survey by using so called Sensi discs from Oxoid. According to these results LMG P-17806 was resistant against aztreonam, cefotaximid, cefoxitin, colistine sulphate, kanamycin, polymyxin B, streptomycin and vancomycin.

LMG P-17806 might thus according to these results be especially interesting for use at the same time as therapy with antibiotics such as vancomycin, trimethoprim as well as several cefixacins, which all are antibiotics with known side effects on the intestine flora and intestine function.

#### THE USE OF THE STRAIN LMG P-17806

##### Example 1 Preparation of Bacterial Concentrate

A frozen bacterial concentrate with 10 billions of LMG P-17806 per gram was prepared in a the way stated above by cultivating the lactobacilli in a substrate of whey added with 1 g yeast extract per litre at a constant pH of 5.5 for 14 hours at 36°C. The bacteria were separated by centrifugation with continuos washing of the centrifugate. The concentrate was frozen in liquid nitrogen and then stored at -30°C until use.

##### Example 2 Preparation of Fermented Milk Product

A product milk was prepared from milk by homogenising the milk, heat-treating it at +95°C for five minutes and tempering it to +37°C. The product milk was grafted with 0.01 % of a commercial, frozen yoghurt culture and 0.5 % of the LMG P-17806 concentrate. The cultures were allowed to grow for six hours at the temperature stated. The milk had then coagulated and the pH decreased to 4.55. The coagulated form was broken and the product chilled to +10°C whereafter it was packed in common plastic cups, which are normally used for yoghurt, and after-cooled in a refrigerating chamber having a temperature of +5°C for one day and night. pH had then decreased to 4.4. The product was then stored at +5°C for up to three weeks.

The content of the strain LMG P-17806 was monitored and as can be seen from the results in Table 7 below, there was a limited growth of the strain LMG P-17806 during the culture and LMG P-17806 survived storing for three weeks at pH below 4.4 very well.

Table 7

The storability of the strain LMG P-17806 in milk products

|              | Content<br>in<br>product<br>milk | Content<br>after<br>cultivation<br>in<br>milk | After<br>storing<br>for 1<br>week | After<br>storing<br>for 2<br>weeks | After<br>storing<br>for 3<br>weeks |
|--------------|----------------------------------|---|-----------------------------------|------------------------------------|------------------------------------|
| Test product | 980                              | 44  | 97                                | 132                                | 118                                |

The contents are given in millions per gram.

The product had a normal appearance with a separation of whey of barely 1 % after storing for fourteen days. The product tasted excellently and had uniform consistency and a fresh, mild flavour. The test product received better judgements than normal yoghurt in an independent consumer survey carried out by an research institute. The product was compared in a consumer survey, in which the testing persons did not know what product was tasted, with a corresponding product without the strain LMG P-17806. The product with LMG P-17806 was preferred by 74% of the persons of the testing panel, and it received the average value of 7.6 in a scale of 9 points, which is significantly higher than the result for common yoghurt. The judgement was slightly more than 1 point higher than a previously known probiotic culture with *Lactobacillus acidophilus*. The results also differ from those previously obtained in comparison between pure yoghurt and mixed products of yoghurt and the probiotic bacteria *Lactobacillus acidophilus* and *Bifidobacterium longum*, respectively. No significant differences could be noted between pure yoghurt

and the respective mixed product in these comparisons.

**Example 3 Suitable Addition of the Strain LMG P-17806**

Experiments were also carried out in order to find out the suitable range for the addition of the strain LMG P-17806.

5 Milk was treated and then added with yoghurt culture according to the above, whereas the addition of concentrate of the strain LMG P-17806 was varied from 0.1% to 0.01%. At such a high content as 5% graft of LMG P-17806 a tang was obtained, which probably originated from components of the graft itself. The 10 change in pH was normal, however, and the appearance of the product was normal. The content of LMG P-17806 in fresh product was 504 million per gram and the content remained at this level during the storage. At the addition of 0.01% LMG P-17806 concentrate the product could not sensrisly be 15 distinguished from common yoghurt and the content of LMG P-17806 was already after one week less than one million per ml, which is the lowest content a product must contain in order to be allowed to state the product to contain a specific probiotic according to proposal to international 20 legalisation.

**Example 4 Preparation on Fermented Special Product**

The experiment was carried out as above except that the milk also was added with 0.4 g yeast extract per litre. The graft was performed with 0.01% of the same yoghurt culture as above, 25 but only 0.1% LMG P-17806 concentrate was added. Incubation was carried out at +34°C for 4 hours whereafter the process was broken off and the product chilled, packed and stored as in Example 3 above. LMG P-17806 grew ten times under these 30 conditions and similarly to the above the bacteria survived well during storage. The results are reported in the Table below.

Table I

## The storability of the specific milk product of the strain LMG P-17806

| Graft | Content<br>in milk<br>product | Content<br>after<br>cultivation | After<br>storing<br>for one<br>week | After<br>storing<br>for two<br>weeks | After<br>storing<br>for three<br>weeks |
|-------|-------------------------------|---------------------------------|-------------------------------------|--------------------------------------|--|
|       | 110                           | 103                             | 114                                 | 104                                  | 107                                    |
| SPUD  |                               |                                 |                                     |                                      |  |

The contents in the table are given in millions per ton product.

The product had a normal appearance with a separation of whey of 1.5% after storing for fourteen days. The product had a good, dry flavour and homogeneous consistency.

Example 5 Preparation of Vegetable Juice Containing the Strain LMG P-17806

A vegetable juice was prepared by mixing carrot concentrate and an orange juice concentrate in equal parts so that pH of the finished mixture became pH 3.9. The mixed beverage was heat-treated and added with 1% and 0.1%, respectively, of LMG P-17806 concentrate. The content of LMG p-17806 became 100 and 10 millions per ml, respectively. The beverages were stored at +7°C for four weeks. The flavour of the product was not affected and no influence by storing or addition of LMG P-17806 was observed. Addition of LMG P-17806 to a content exceeding 50 millions, however, seemed to reduce the decrease in vitamin C. In a product without LMG P-17806, like in the product having 10 millions bacteria per ml, the content of vitamin C decreased from 25 mg/100g, before the storage to 16 mg/100g after four weeks. With the addition of 100 millions LMG P-17806 ml the content of vitamin C only decreased to 14 mg/100g, i.e. more than 50% lower decrease in the content of Vitamin C. LMG P-17806 survived well and the content of LMG P-17806 after four weeks was only about 40% lower than in fresh

product independent of the amount added.

In another experiment the same fruit beverage was added with 5% LMG P-17806. This gave a tang, which grew worse during storage time.

5

#### Example 6 Preparation of Pap Powder

Pap powder is manufactured in the common way. LMG P-17806 concentrate according to the above is lyophilized after mixing with corn starch and stored after being packed in an oxygen-tight wrapping at a temperature of -20°C. The lyophilised preparation contained 61 billions LMG P-17806 per gram. The pap powder was dried-mixed with 0.03% LMG P-17806 and packed in an atmosphere of nitrogen gas in an oxygen tight wrapping. The powder was stored initially for three months at +12°C and thereafter at a room temperature for additional four months. The content of LMG P-17806 is apparent from Table 9 below. The pap powder gave good possibilities for survival to LMG P-17806 and therefore it is possible to prepare, for instance, baby food products having a high content of LMG P-17806.

10

Table 9

| Lyophilized preparation | Powder before addition | Powder after addition | After 3 months | After 7 months |
|-------------------------|------------------------|-----------------------|----------------|----------------|
|                         | 61,000                 | <0.0001               | 20             | 18             |

The content in the Table above are given in millions per gram powder.

The pap powder was dissolved in 9 parts of water of +50°C and the content of LMG P-17806 determined. The content was 1 million LMG P-17806 per ml beverage in the case that six months' old powder was used. The pap could not be distinguished from common product. After storing for one night in room temperature, however, the pap added with LMG P-17806

15

tasted slightly acid and had pH of 5.7.

In a second experiment the effect of the addition of 1 and 0.0011, respectively, of lyophilized LMG P-17806 preparation was examined. The contents thereof in the pap powder after the addition were 571 and 0.8 millions per gram respectively.

The powders were stored in a corresponding way and examined after storage for 5 months. After dissolution as above the content in the prepared pap was 600 millions, respectively for the low addition less than 10,000 per ml. With the high addition a slightly acid flavour was noted already after stirring for four hours of the pap at body temperature.

**Example 7 Preparation of Dried Powder for Use as "Medical Food"**

A lyophilised LMG P-17806 concentrate was mixed with different amounts of corn starch in the proportions in 1:1, 1:9, 1:99 and 1:999, respectively. The mixed powders were stored in small sachets with 1 to 100 g per sachet. The material of the sachets was impervious to oxygen and water vapour. The sachets were stored in freezer, refrigerator and room temperature, respectively. The content was then used as an additive to beverage by mixing the powder with the beverage before the beverage being consumed. The corresponding survival as in example 6 above was obtained. The powder with the proportions 1:1 was also packed in gelatin capsules with 0.4 g per capsule. The number of bacteria per capsule was 10 billions. The capsules were blister-packed in a material with good barrier properties against oxygen and water vapour. The capsules were stored in the same way as stated above and corresponding good survival results were obtained.

**Example 8 Preparation and Use of Bacterial Mixes**

LMG P-17806 can be mixed with other lactobacilli without inhibiting them. LMG-17806 does not seem to form any substances which are inhibiting other lactobacilli or lactococci where LMG P-17806 are co-cultivated with yogurt

5 cultures, sour milk cultures with *lactococci* or other lactobacilli species such as *L. acidophilus*, *L. fermentum* or *L. rhamnosus*. Experiments with mixtures of lyophilized preparations containing all these bacteria have indicated unchanged storage properties whether mixing was carried out with corn starch or with pap powder. After the solution of the pap powder and a storage for 12 hours at body temperature and 10 hours at room temperature no negative influence by LMG P-17806 could be traced either on the total content of 10 lactobacilli or on the content of either of the bacteria.

15 In an experiment to produce probiotic sour milk 0.5% of sour milk culture and 0.1% of lyophilized concentrate of each of *L. acidophilus* NCFm 1746, *L. fermentum* K12 and LMG p-17806 were added to normal treated product milk. However, only 0.1% K12 was added, because higher additions gave rise to tang and to a bad coagulum. Milk was stored for 19 hours at room temperature. Then the pH thereof was 4.50. The milk was cooled to +10°C, packed and stored at +6°C or up to 14 days. The results are evident from the following table 10.

20

Table 10

| Lactic acid bacteria  | Before cooling | After 5 days | After 14 days |
|-----------------------|----------------|--------------|---------------|
| Lactococci            | 730            | 810          | 580           |
| <i>L. acidophilus</i> | 135            | 142          | 131           |
| <i>L. fermentum</i>   | 12             | 14           | 8             |
| LMG P-17806           | 112            | 126          | 109           |

The contents are in millions per ml of the different bacteria.

25 80 healthy test subjects were given the products to eat in connection with a conference journey to Istanbul in Turkey. The group was divided into two, one eating a sour milk according to the above with only LMG P-17806 whereas the other ate the probiotic sour milk with three different probiotic

strains. The test subjects were on the conference place for 3 days. They started to eat the products two days before departure to the conference place and continued to eat for four days after home-coming. The products were eaten as 3 snacks evenly spread during the day with 150 g/meal. All test subjects except 2 persons of the sour milk group declared that they ate the product according to the scheme. In an inquiry they were asked to state discomfort from the gastrointestinal tract in the form of stomach pains, tensions, diarrhoea or constipation on a scale of 0-3 degrees. Apart from diarrhoea there was a difference so far that 30 of the test subjects which only had eaten LMG 1-1780 stated that they had had serious or very serious diarrhoea during at least two days whereas this frequency only was 12 in the group which ate the probiotic sour milk.

Thus it seems as if a mix of several different lactobacilli might be still more effective than only one single strain of bacteria. This can be due to the fact that the lactobacilli administered had different properties.

## C L A I M S

1. Strain of *Lactobacillus* useful as probiotics in food and naturopathic medicines and which is resistant in-vitro against hydrochloric acid and gastric juice and tolerates bile salts without deconjugating them, whereas a strong assimilation is occurring and which has a good survival at passage through the stomach and the gastrointestinal tract and which strain is growing optimally at about 37°C, **characterised** in that the strain is *Lactobacillus paracasei* subsp. *paracasei*, which is a Gram-positive, homofermentative, rod-shaped bacterium capable of producing L-lactic acid and in that it contains three plasmids having a size of 2.1, 4.3 and 9.1 Kb, respectively.
- 10 2. Strain according to claim 1, **characterised** in that it contains 44% GC.
- 15 3. Strain according to claim 1 or 2, **characterised** in that it has been isolated from samples from the gastrointestinal micro-flora of humans.
- 20 4. Strain according to claim 1, 2 or 3, **characterised** in that the strain is fermenting ribose, adonitol, galactose, glucose, fructose, mannose, sorbose, mannitol, sorbitol, N-acetyl-glucosamine, esculin, cellobiose, maltose, lactose, sucrose, trehalose, inulin, melezitose, D-turanose and D-tagatose.
- 25 5. Strain according to claim 1, 2, 3 or 4, **characterised** in that it is provided as a concentrate in the form of frozen or lyophilized powder.
- 30 6. Composition of a strain according to any of the preceding claims for use in food and/or as naturopathic medicines, **characterised** in that the strain *Lactobacillus paracasei* subsp. *paracasei* is added to a milk product, a fermented milk product, fruit or vegetable beverage such as vegetable juice, citrus fruit juice or a juice of another fruit or vegetable in a content of between 0.001% and 5%, preferably 0.01 and 1%.

7. Composition of a strain according to any of claims 1-6 for use as a probiotic in food and/or naturopathic medicines, **characterised** in that the strain *Lactobacillus paracasei* subsp. *paracasei* according to the invention is co-cultivated with yoghurt cultures and sour milk cultures with *lactobacilli* or other *Lactobacillus* species such as *L. acidophilus*, *L. fermentum* or *L. rhamnosus*.

8. Composition according to claim 7 or 9, **characterised** in that the strain *Lactobacillus paracasei* subsp. *paracasei* according to the invention is present in a content of between 1 million and 15,000 millions living bacteria per gram of probiotic product.

9. Product containing the strain *Lactobacillus paracasei* subsp. *paracasei* according to any of claims 1-4, **characterised** in that the product consists of

15 a milk product, a fermented milk product, a vegetable or fruit beverage or pap powder all containing the strain *Lactobacillus paracasei* subsp. *paracasei* in a content of  $5 \times 10^5 - 5 \times 10^9$ , usually  $1 \times 10^5 - 10^9$  living bacteria, corresponding to 0.0005-0.5% of the product;

20 or a concentrated naturopathic medicine ("Medical Food" wherein the strain *Lactobacillus paracasei* subsp. *paracasei* is present at a content of 1,000-100,000 millions living bacteria corresponding to 0.001-100% of the product.

25 10. Product according to claim 9 containing the stated content of bacteria, **characterised** in

30 that in case of food products it further contains a small proportion, e.g. 0.001-0.1%, of yeast extract or other substances which contribute to growth or survival of *Lactobacillus paracasei* subsp. *paracasei* in the product,

and that in case of naturopathic medicines it contains substances of importance for survival of the bacteria and/or residues of the culture substrate.

35 11. Product according to claim 9 or 10, **characterised** in that the strain *Lactobacillus paracasei* subsp. *paracasei* is

provided as a concentrate in frozen or lyophilized condition for mixing in direct connection with consumption occasion into a milk product, a fermented milk product, a vegetable or fruit beverage, in pap powder or in a concentrated naturopathic medicine (so-called "Medical Food").

12. Product containing a strain of *Lactobacillus paracasei* subsp. *paracasei* according to any of claims 1-5, **characterised** in that it is used

10 for children for the purpose of alleviating atopic problems;

for elderly persons in order to correct altered microflora caused by normal changes by age or an altered secretion of hydrochloric acid;

15 and for persons in general in order to normalise the intestinal flora in which case the content of clostridia bacteria is decreasing, bifidobacteria is increasing and high contents of coliformic bacteria are decreasing.

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE 98/02263

## A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C12N 1/20 // (C12N 1/20, C12R 1:225)

According to International Patent Classification, IPC or to the national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C12N, A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE, DK, FI, NO classes as above

Electronic data base consulted during the international search: name of data base and, where practicable, search terms used

WPI, CA, BIOSIS, MEDLINE

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category <sup>1</sup> | Citation of document, with indication, where appropriate, of the relevant passages   | Relevant to claim No. |
|-----------------------|--|-----------------------|
| X                     | Dialog Information Services, file 5, BIOSIS PREVIEWS, Dialog accession no. 13333687, Biosis no. 99333687, Mercenier A et al: "Development of lactic acid bacteria as live vectors for oral or local vaccines"; & Advances in Food Sciences 18 (3-4). 1996. 73-77<br><br>-- | 1-12                  |
| X                     | Dialog Information Services, file 5, BIOSIS PREVIEWS, Dialog accession no. 11493893, Biosis no. 98093893, Harty D W S et al: "Pathogenic potential of lactobacilli"; International Journal of Food Microbiology 24 (1-2). 1994. 179-189<br><br>--                          | 1-12                  |

 Further documents are listed in the continuation of Box C. See patent family annex.

- \* Special categories of cited documents:
- A document defining the general state of the art which is not considered to be of particular relevance
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- & document member of the same patent family

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## INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE 98/02263

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category | Citation of document, with indication, where appropriate, of the relevant passages   | Relevant to claim No. |
|----------|--|-----------------------|
| X        | WO 9709448 A1 (OULUTECH OY), 13 March 1997 (13.03.97), see page 3, line 32 - page 4, line 2, page 7, lines 25-34<br>--   | 1-12                  |
| A        | Chemical Abstracts, Volume 125, No 23, 2 December 1996 (02.12.96), (Columbus, Ohio, USA), Klein Guenter et al, "Total soluble cytoplasmatic protein patterns of Lactobacillus rhamnosus and Lactobacillus paracasei from different habitats", page 557, THE ABSTRACT No 296749m, Mikrooekol. Ther. 1995, 23, 179-187<br>-- | 1-12                  |
| A        | Dialog Information Services, file 5, BIOSIS, Dialog accession no. 11400418, Biosis no. 199800181750, Savova T et al: "Lactobacillus casei: Survival in the gastrointestinal tract and biostimulating activity"; & Zhivotnov"dni Nauki 33 (7-8):p55-57 1996<br>--   | 1-12                  |
| A        | Dialog Information Services, file 5, BIOSIS, Dialog accession no. 02137076, Biosis no. 000063052076, Gilliland S E et al: "De conjugation of bile acids by intestinal lactobacilli"; & Appl Environ Microbiol 33 (1). 1977 15-18<br>--<br>-----  | 1-12                  |

## INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

02/03/99

PCT/SE 98/02263

| Patent document cited in search report | Publication date | Patent family member(s)                    | Publication date                 |
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| WO 9709448 A1                          | 13/03/97         | AU 6877396 A<br>FI 102298 B<br>FI 954194 A | 27/03/97<br>00/00/00<br>08/03/97 |